MITIGATION OF *PRYMNESIUM PARVUM* BLOOMS BY CLIPPER HERBICIDE

A Thesis

by

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ABSTRACT

The harmful algal species *Prymnesium parvum*, also known as golden algae, is toxic to many forms of aquatic life. When it blooms it kills fish and other aquatic organisms and can have long-lasting impacts on the ecosystem where it blooms. Many forms of treatments are employed to control *P. parvum*, including chemical methods. Flumioxazin, the active ingredient of Clipper herbicide, has been effective at controlling *P. parvum* in small-scale situations. A concentration gradient of Flumioxazin including 50, 100, 200, and 400μg/L was tested in its commercial form of Clipper in a laboratory setting to determine whether it would be effective, considering its supposed inactive and inert ingredients. Varying conditions at the time of application were also examined to aid in decision making processes where Clipper is being considered as a means of control. The conditions examined were season (fall and winter of the southeastern United States) and growth stage (log and stationary) of *P. parvum*. Through the use of cell counts by light microscopy and chlorophyll-*a* fluorescence, Clipper was determined to control *P. parvum* in winter season, but not in fall season, and to be more effective when applied in *P. parvum*'s log growth phase than in its stationary growth phase. This is likely due to *P. parvum* having an optimum growing temperature closer to the fall season than the winter season of the southeastern United States and that the growth rate of *P. parvum* in its stationary phase is already low in comparison to its growth rate in log growth.

CONTIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Dr. Daniel Roelke, who was my advisor, and Dr. Delbert Gatlin of the Wildlife and Fisheries Department of and Dr. Thornton of the Department of Oceanography.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

When algal blooms negatively affect organisms in their environments, they are called harmful algae blooms (HABs). They can be harmful by releasing toxins, inducing hypoxic conditions and altering ecological food webs. (Smayda 1997; Paerl & Paul 2012). They seem to be occurring with increasing frequency and duration (Smayda 1990; Hudnell 2010; Brooks et al. 2016). Many factors contribute to the development of HABs.

Anthropogenic contributions include eutrophication (Anderson et al. 2002), contamination and resource use from aquatic ecosystems (Brooks et al. 2016). Even introduction of invasive organisms such as zebra mussels (*Dreissena polymorpha)* have contributed to toxic algal bloom success (Vanderploeg et al. 2001). Natural processes are just as important to consider as anthropogenic contributions (Sellner et al. 2003). For example, circulation in the oceans and estuaries as well as river flows affect the distribution of algae populations (Sellner et al. 2003). The short-term and long-term effects that HABs have along with the factors that control blooms vary widely (Anderson 2009). It is thus reasonable to conclude that there cannot be a single solution to controlling HABs across the globe. Instead, we should seek to develop management strategies on bases of specific organisms and situations.

A particularly harmful blooming phytoplankton is *Prymnesium parvum*, a haptophyte that is typically found in estuarine and marine environments, but also in brackish inland waters. It is a mixotrophic organism acquiring energy autotrophically, but also employing heterotrophic strategies under certain conditions. Toxins from *P. parvum* suppress phytoplankton competitors and immobilize prey. In consequence, fish and other aquatic organisms are harmed and killed by the toxin (Graneli 2006; Brooks et al. 2010; Remmel & Hambright 2012). The use of its toxin to neutralize competitors and predators while immobilizing prey, along with its ability to shift nutritional gathering strategies and being resistant to its competitors' allelopathic chemicals all contribute to *P. parvum's* ability to successfully bloom (Roelke et al. 2010). When *P. parvum* blooms, it is able to produce acute effects

such as killing fish and other aquatic organisms as well as chronic effects where the size structure and abundance of fish have been negatively impacted even after the bloom resolves (VanLandeghem et al. 2013).

In Texas, *P. parvum* was first confirmed to be present and problematic in the Pecos River (Southard et al. 2010). It is uncertain whether *P. parvum* was introduced into Texas or if it had been present but remained undetected because no one was looking for it. Regardless of its origins, *P. parvum* blooms have been a sporadically reoccurring event in Texas. As of 2008, these events have killed over 34 million fish with an economic cost estimated at almost 13 million USD (Southard et al. 2010). It is likely that these numbers are now much higher considering blooms have continued to the present day. Blooms of *P. parvum* have occurred in 23 states in the U.S., mostly in the south, but certainly not restricted there (Roelke et al. 2016).

The factors leading to blooms of *P. parvum* are still largely debated, but there is much agreement that some factors at least play a role. Temperature likely plays a role as *P. parvum* tends to bloom in cooler, winter months in the southern U.S. (Hambright et al. 2010; Roelke et al. 2010; Southard et al. 2010). Conditions consisting of limited nutrients leads to blooms and increased toxicity (Roelke et al. 2007; Uronen et al. 2005). Salinity likely plays a role in *P. parvum* bloom formation. Higher salinity is needed to support *P. parvum* blooms. Minimum estimated practical salinity units have been recorded for individual lakes and rivers, below which blooms will not form. *P. parvum* blooms require salinities greater than 1.5 psu in Lake Possum Kingdom, TX. In Lake Whitney and Lake Granbury, TX salinities greater than 0.5 psu are needed for *P. parvum* to bloom. (Roelke et al. 2011)

There are several methods that have been suggested to control *P. parvum* blooms. These strategies can either be reactive, where they are used to mitigate a bloom after it has formed, or proactive, where the strategy is used to prevent or at least curtail blooms by anticipating, preparing and acting before the bloom has occurred. The most effective management will include strategies

from both methods of management. Proactive management should be actively implemented in attempts to prevent blooms in the first place. Continued research into the factors that increase the chances a bloom will occur may produce useful knowledge for the formation and implementation of proactive strategies. It will continue to be important to develop effective reactive strategies to deal with blooms that occur in spite of diligent proactive management.

VanLandeghem et al. (2013) suggested the use of improving water quality by reducing nutrients and increasing water inflow to limit *P. parvum* blooms. Roelke et al. (2016) added that they may even be the only strategies to control *P. parvum* blooms system wide. However, they also considered that the costs to reduce nutrient loads would likely exceed the economic benefits of reducing *P. parvum*, and that it would be impractical due to water quantity limitation to increase water inflows in many areas.

Aside from being impractical in many instances, reactively managing *P. parvum* through reducing nutrients after *P. parvum* has been established does not yield the expected decrease in number and in fact introduces a management paradox. Conditions of limited nutrients could provoke *P. parvum* to become more toxic with reduced nutrients as mentioned previously allowing it to extend its bloom length. It is possible to reduce toxicity through nutrient enrichment, but as *P. parvum* cell density increases and nutrients become depleted, toxin production is stimulated again (Brooks et al. 2011). Additionally, nutrient enrichment can be detrimental to sensitive aquatic species through higher pH and increased unionized ammonia (Kurten et al. 2007).

The treatment of *P. parvum* blooms in natural settings has not been attempted. Roelke et al. (2016) suggested the alternative of managing smaller areas of water within the system to create refuges, thereby allowing a quicker recovery of the system at the termination of the *P. parvum* bloom. Chemical and hydraulic treatments in smaller bodies of water can be used in finding practical ways to control blooms in these larger, natural settings (Roelke et al. 2016).

Hydraulic treatments to control *P. parvum* blooms take the form of hydraulic flushing. Hydraulic flushing involves the use of low nutrient water being flushed into the water affected by *P. parvum* at a rate higher than the growth rate of *P. parvum*. The idea is that *P. parvum* populations will be displaced and the environmental conditions facilitating production of toxins that contribute to blooms will become disrupted. With higher and more continual flushing this has worked well when deep water is available to pump from into coves of water containing blooms. (Hayden et al. 2012; Lundgren et al. 2013). Flushing also has significant impacts on the phytoplankton community composition (Hayden et al. 2012). The displacement of cells selects for the species that reproduce quickly. *Prymnesium parvum* reproduces slowly, especially in the winter when concerns for bloom are prevalent, allowing flushing to be an effective control. Lundgren et al. (2013) and Hayden et al. (2012) conducted these studies in Lake Granbury, Texas. In smaller, shallower water bodies, it would not be possible to utilize this method without using external sources of water, which may not be available or economically beneficial. A further limitation is that while winter lasts, *P. parvum* populations might return to previous bloom levels when flushing ceases. While other benefits of hydraulic flushing were seen, it is important to understanding that persistent flushing may be required to control *P. parvum* blooms and thus may not be feasible in some systems.

Broad-spectrum chemical treatments have been implemented in an effort to control *P. parvum* and its resulting blooms. There are challenges involved with these to make applications that effectively control *P. parvum* and its toxins while avoiding the introduction of toxicity and secondary effects to other phytoplankton species and aquatic life.

Potassium permanganate may be used to reduce toxicity of the water (Barkoh, 2010). It acts as an oxidizer of organic and inorganic substances. It is able to kill bacteria and phytoplankton. After its permanganate ion is oxidized it becomes manganese dioxide which is relatively nontoxic. The amount needed to control the desired organism is dependent on the potassium permanganate demand which is determined by the amount that is taken up and converted into manganese dioxide. It is

recommended to apply 2 to 4 mg/L above the potassium permanganate demand to control *P. parvum* blooms and its ichthyotoxicity (Barkoh, 2010).

Ammonium sulfate (Barkoh et al. 2003) has been used to kill *P. parvum* directly. Ammonium sulfate lyses *P. parvum* cells with only negligible effects on other life in some cases (Shilo & Shilo 1953) and harmful effects on some fish species such as red drum (*Sciaenops ocellatus*), rainbow trout (*Oncorhynchus mykiss*) and sunshine bass (*Morone chrysops* × *M. saxatilis)* in other cases (Barkoh et al. 2004). Barkoh et al. (2004) states that the levels of ammonia toxicity that they tested may have chronic effects on the fish studied. So the effects on fish may not be seen during short-term experiments. The effectiveness of ammonium sulfate fluctuates with the level of pH. At higher pH, it is more effective, but at lower pH it can be ineffective at controlling *P. parvum* (Shilo & Shilo 1953).

Copper algaecides (Southard et al. 2010) also have been used to kill *P. parvum.* Copper is an important cofactor to oxidative phosphorylation and photosynthesis, but at higher levels than is optimum, it kills phytoplankton (Rouco et al. 2014). Copper algaecides with application rates of 0.2 to 1.0 mg/L have been shown to be toxic to fathead minnows (*Pimephales promelas*) and brook trout (*Salvelinus fontinalis*) at those levels in laboratory studies (Closson & Paul 2014). However, in field studies these results are not replicated; instead fish do not experience mortality from applications of the copper algaecides tested by Closson & Paul (2014). Copper treatments have effects on overall algal biomass and community structure especially in combination with zinc (Atazadeh et al. 2009).

Flumioxazin has been studied and shown to be effective at targeting and mitigating *P. parvum* (Umphres et al. 2012) with minimal effects on *Lepomis macrochirus,* known commonly as bluegill (Umphres et al. 2013). That evaluation was done at a temperature of 12°C. In the interest of developing management strategies, considering proactive and reactive needs, it would be advantageous to know how well the product Clipper works in controlling *P. parvum* with its supposedly inert and inactive ingredients and in a wider variety of situations, e.g., warmer temperatures when blooms are just initiating. In other words, because *P. parvum* blooms form

primarily in the winter it is important to understand how the chemical works in winter conditions allowing for reactive methods to be used. Understanding its efficacy in fall conditions would also be of importance because it might be that the chemical could be used along with monitoring of temperature in a proactive strategy so that the chemical could be applied before blooms occur but conditions make them likely.

As a protox inhibitor, flumioxazin acts by blocking the production of protoporphyrinogen oxidase which is an enzyme necessary to synthesize chlorophyll (Matzenbacher et al. 2014). Temperature is directly related to the rate at which chemical reactions take place (Matzenbacher et al. 2014). Conceptually, Clipper's efficacy at controlling *P. parvum* blooms should be enhanced with higher temperatures. That idea was the hypothesis of the current study.

In this study, I determined the ability of the chemical product Clipper to control *P. parvum* blooms considering season, *P. parvum* growth phase, and the concentration of its active ingredient, flumioxazin. Specifically, I exposed cultures of *P. parvum* in both log growth phase and stationary phase at 16° C (to simulate fall conditions) for 12 days to a chemical concentration gradient, then repeated the same experiments but at 12° (to simulate winter conditions). This experimental design yielded three controlled independent variables: temperature, growth phase, and Clipper concentration, where the two different temperatures enabled a test of the effect of season, the two phases of growth were considered to test the idea that application of a chemical in earlier growth phases may suppress the bloom more efficiently as suggested by Umphres et al. (2012), and a gradient of chemical concentration enabled a test of the most effective dosages.

2. MATERIALS AND METHODS

The experiment consisted of four chemical screenings. Each screening was done to allow for determination of Clipper's efficacy at controlling P. parvum and mitigating blooms considering the dependent variables (season, *P. parvum* growth phase, and Clipper concentration). For the first screening, temperature was 16°C and *P. parvum* was in log growth with a chemical gradient applied. Temperature was 16°C and *P. parvum* was in stationary growth with a chemical gradient applied for the second screening. For screenings 3 and 4, screenings 1 and 2 were repeated but at 12°C. Winter temperatures were used because it mimicked winter conditions based on Umphres et al. (2012). Fall temperatures are a representative temperature of fall in the southeastern United States.

The *P. parvum* monoculture (UTEX LL 2979, Culture Collection of Algae at The University of Texas at Austin) used in this study was maintained by re-inoculating monthly into fresh F/2 media (Guillard 1975) added to reverse osmosis water at a 10:1 ratio. This enabled the culture to reach stationary growth phase each month prior to re-inoculation.

To experimental units, inocula were made from *P. parvum* monocultures. Each screening comprised 15 experimental units, which were maintained in a LI15 SHEL LAB diurnal plant growth chamber (120v) for 12 days with a 12hour: 12hour dark: light cycle. Light was provided through fluorescent bulbs at 115 µmol m^{-2} s^{-1}. The positions of experimental units were rotated daily to give equal light exposure to each unit throughout the screening. Experimental units were grown in 125 mL Erlenmeyer flasks with parafilm stretched over the tops as dust covers to maintain an axenic experiment. At initiation, the experimental units contained 45ml of fresh F/2 media with varying concentrations of Clipper dissolved into it and 5 ml *P. parvum* inoculum growing in log phase. Experimental units comprising screenings that employed cultures in stationary growth phase contained 45 ml of spent media with varying concentrations of Clipper dissolved into it and 5 ml *P. parvum* inoculum in stationary phase. The spent media was made by filtering *P. parvum* cells from

the inoculum source using Whatman GF/F filters (0.7μm pore size). Cultures were acclimated to experimental temperatures for several weeks prior to the experiments

There were five treatments for each screening which differed from each other by the concentration of flumioxazin that was present at the beginning via Clipper additions. These were a control with no Clipper, and flumioxazin additions via Clipper at concentrations of 50, 100, 200 and 400 μg/L. These concentrations were selected to give a wide gradient that included concentrations at which flumioxazin is known to be effective at mitigating *P. parvum* blooms without added Clipper ingredients (Umphres et al. 2012). Each treatment was performed in triplicate (15 experimental units per screening).

The response variables of interest were the chlorophyll-*a* concentrations and *P. parvum* population cell densities. For chlorophyll-a, on the first day and then every other day, *in-vivo* fluorometry was done using standard methods (Yentsch & Menzel 1963) on 4 mL from each experimental unit using a Turner Design 10-AU fluorometer. This method was used to estimate the chlorophyll-*a* content in units of relative fluorescence. This method has limitations however, in that cell-packaging effects are unaccounted for, which can lead to under-estimations of chlorophyll-*a*. For cell density, following fluorometry, the 4 mL collected from each experimental unit were preserved in glutaraldehyde (5% v/v) for use in cell counts.

Cell counts were done using a light microscope on improved Neubauer hemocytometers whose performance and accuracy is documented by Mahmoud et al. (1997). Limitations to using hemocytometers for cell counts are that large cells may not be contained in the small chambers and only small samples may be held. The improved Neubauer is 0.1 mm deep while *P. parvum* range from 8-15 micrometers (0.008-0.015mm) in length and 4-10 micrometers (0.004-0.01mm) in width (Manning & La Claire 2010), so *P. parvum* are of an appropriate size for counting by this method.

To reduce the loss of accuracy in counting small sample sizes, at least six chambers were counted and at least 200 cells or more per sample were counted. The exception was when no cells were found after counting six chambers in a row, it was determined that the density was below a reasonable detection threshold and the count up to that point was used to calculate cell density.

Data were analyzed using sequential 3-way Analyses of Variance (ANOVA) to examine the effects of Clipper treatments on the effects of temperature, growth stage, and Clipper concentration on the *P. parvum* per capita growth rate and on changes in chlorophyll-a concentrations. The data were then separated along lines of temperature differences and used to conduct 2-way ANOVAs to analyze the effects of growth stage and Clipper concentration on the *P. parvum* per capita growth rate and on changes in chlorophyll-a concentrations. For these analyses, per capita growth rates were calculated using:

$$\mu(N) = \frac{(N_t - N_0)}{tN_0}$$

$$\mu(Chl) = \frac{(Chl_t - Chl_0)}{tChl_0}$$

where N_t and N_0 were the P. parvum cell densities at the time of sampling and in the initial condition (cells mL^{-1}), Chl_t and Chl_0 were the chlorophyll a concentrations at the time of sampling and in the initial condition ($\mu g/L^{-1}$), and t was the time of sampling (d).

ANOVAs were done using the software, SPSS version 23 and were followed by a Tukey's and Games-Howell tests.

3. RESULTS

3.1 Results overview

Cell density and relative fluorescence is summarized in figures 1 and 2 and in tables 1 and 2. Per capita growth in *P. parvum* populations and chlorophyll-a changes support the same notion: that in fall temperatures, the action of Clipper was not significant ($p>0.05$) and makes no changes to the population, but that in winter temperatures, Clipper concentration was significant ($p<0.05$) in the outcome of the *P. parvum* population growth and chlorophyll-a concentration.

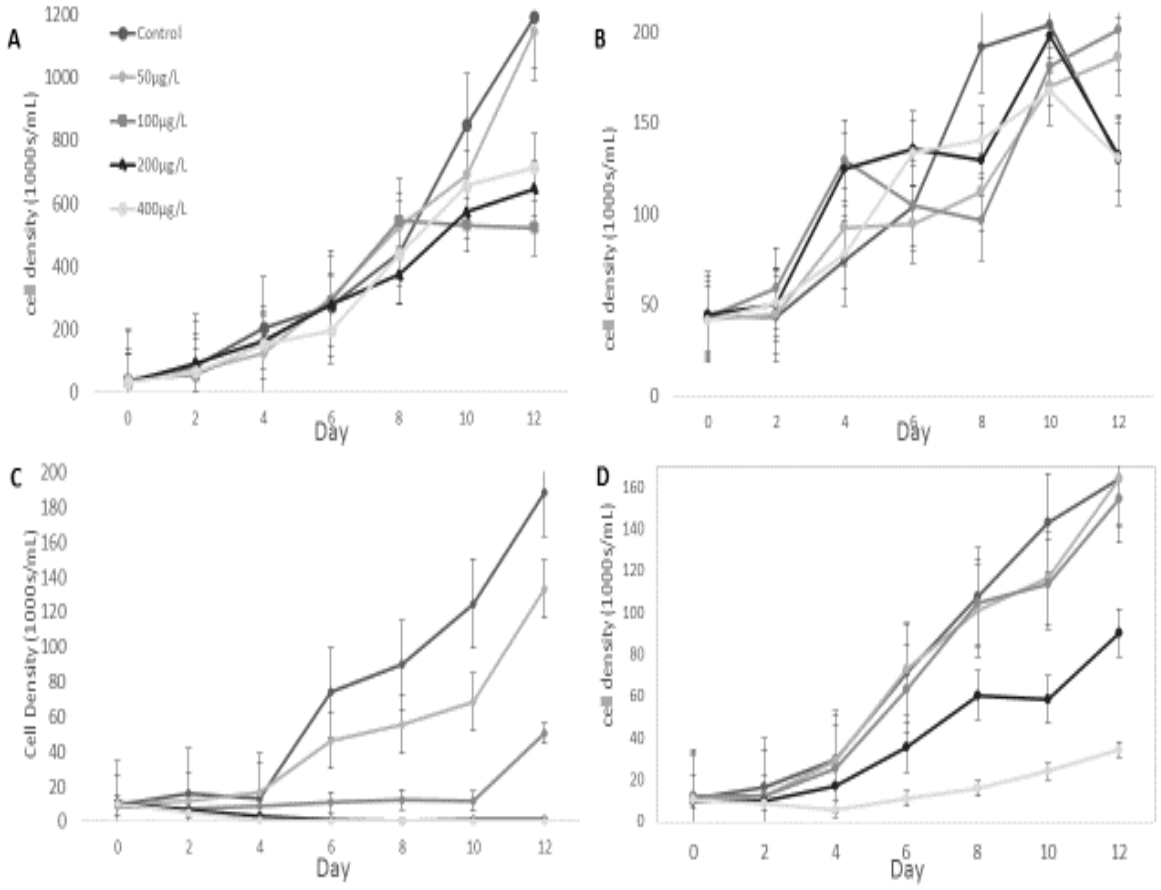

Fig. 1.

Effects of various concentration of flumioxazin via Clipper on the cell densities of *P. parvum.* (A) 16°C (fall) in log growth stage, (B) 16°C (fall) in stationary growth stage, (C) 12°C (winter) in log growth stage, and (D) 12°C (winter) in stationary growth stage.

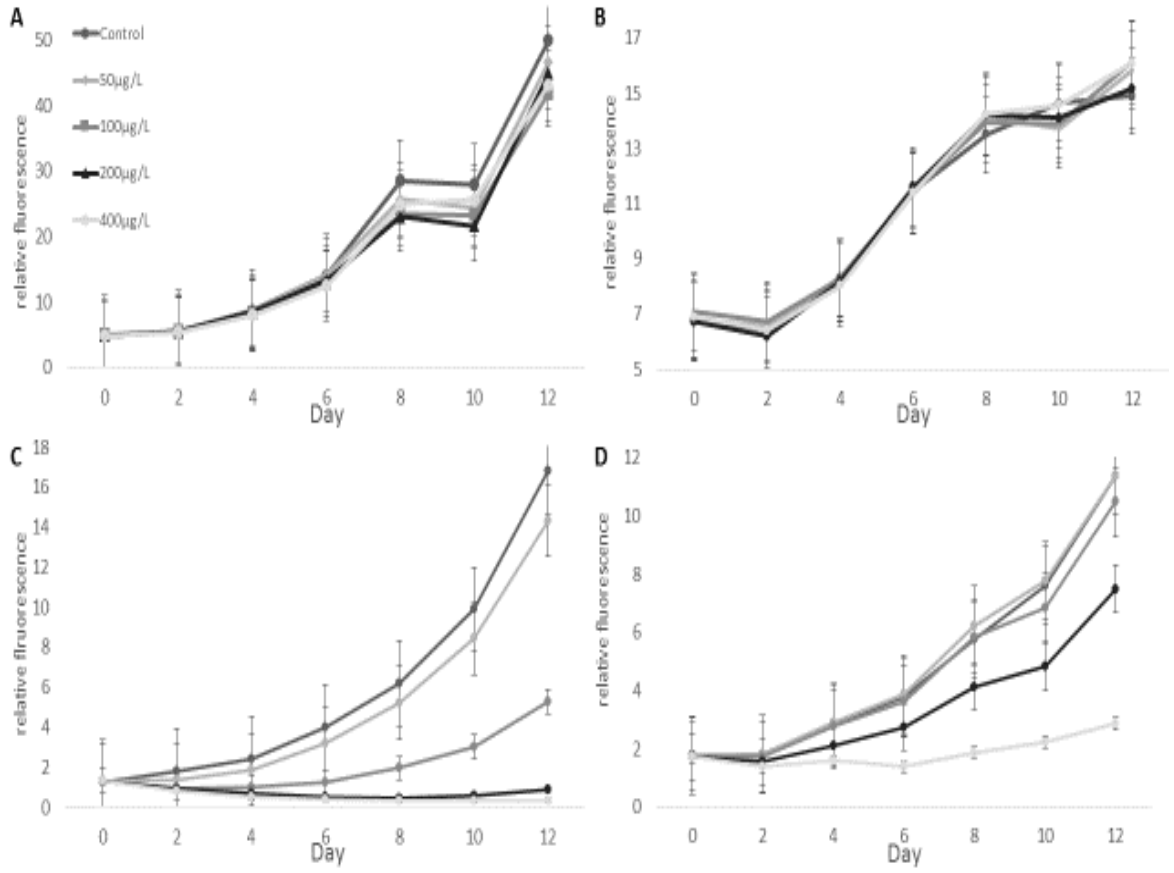

Fig. 2.

Effects of various concentration of flumioxazin via Clipper on the chlorophyll-a concentration of *P. parvum*. (A) 16°C (fall) in log growth stage, (B) 16°C (fall) in stationary growth stage, (C) 12°C (winter) in log growth stage, and (D) 12°C (winter) in stationary growth stage. On Day 8 of 4A, the fluorescence is likely lower than depicted. The samples were at room temperature rather than at their experimental temperatures for much longer than intended due to difficulties experienced this day.

	Fall		Winter	
	Log	Stationary	Log	Stationary
Control	0.43±0.24	0.14±0.23	0.52±0.87	0.3±0.22
50 µg/L	0.40±0.18	0.16±0.20	0.31±0.33	0.34±0.31
100 µg/L	0.34±0.36	0.19±0.28	0.3±0.65	0.31±0.29
200 µg/L	0.37±0.32	0.15±0.32	0.16±0.98	0.25±0.21
400 µg/L	0.37±0.26	0.12±0.17	-0.08±0.58	0.15±0.22

Table 1. Average per capita daily growth of *P. parvum* over 12 days. *P. parvum* was exposed to varying levels of Clipper herbicide with standard deviation (n=3).

	Fall		Winter	
	Log	Stationary	Log	Stationary
Control	0.26±0.20	0.079±0.083	0.27±0.06	0.19±0.10
50 µg/L	0.25±0.19	0.081±0.082	0.26±0.13	0.19±0.11
100 µg/L	0.23±0.17	0.079±0.080	0.16±0.19	0.18±0.13
200 µg/L	0.25±0.20	0.079±0.098	-0.01±0.17	0.15±0.12
400 µg/L	0.24±0.18	0.082±0.089	-0.09±0.08	0.05±0.11

Table 2. Average per capita daily change of fluorescence of *P. parvum* over 12 days. *P. parvum* was exposed to varying levels of Clipper herbicide with standard deviation (n=3).

3.2 Effects on per capita *P. parvum* growth

In winter temperatures, using flumioxazin concentrations via Clipper greater than 200 µg/L caused negative per capita growth of *P. parvum* when applied while *P. parvum* in log growth. When

P. parvum was in stationary growth, Clipper concentrations greater than 200 μg/L flumioxazin were necessary to have a significant ($p<0.05$) effect on *P. parvum* growth. (fig. 3)

The three-way interaction between the tested independent variables (Clipper concentration x season x growth stage) was not significant ($p>0.05$) in effect to the per capita growth. The greater effects were by interactions of season and growth stage as well as interactions between season and Clipper concentration for the first 10 days of the screening. By the twelfth day, the interaction between season and Clipper concentration was not significant ($p>0.05$), but there were significant ($p<0.05$) effects by interactions between season and growth stage and between growth stage and Clipper concentration. (Tab. 3)

The results of the 3-way ANOVAs led me to conclude that season by means of differences in temperature had the most consistent significant effect across the concentration gradient. It is thus useful to separate the data along the lines of season to look deeper into what other effects impacted these results beyond temperature.

	Day					
	2	4	6	8	10	12
Interactions and Effects						
Temp*GS*Cconc	0.255	0.441	0.074	0.131	0.993	0.518
GS*Cconc	0.152	0.38	0.414	0.825	0.05	0.005
Temp*Cconc	0.044	0.033	0	0.006	0.004	0.069
Temp*GS	0	0	0	0	0	0
Temp	0	0	0.003	0	0	0.002
GS	0.002	0.05	0.003	0	0	0
Cconc	0.117	0.214	0	0.002	0	0

Table 3. 3-way ANOVA *P. parvum* per capita growth. Values listed are the p values for per capita growth from the treatment with Clipper to the day listed at the top of the column (ti-t0). Highlighted squares are the interactions or effects considered significant (p value < 0.05 and prioritizing interactions before effects)

2-way- analysis of variance revealed that growth stage was the only significant effect in fall temperatures, but that in winter temperatures, growth stage and Clipper concentrations had varying significance to the per capita growth of *P. parvum* over time and settled on an interaction between them. (Fig.3)

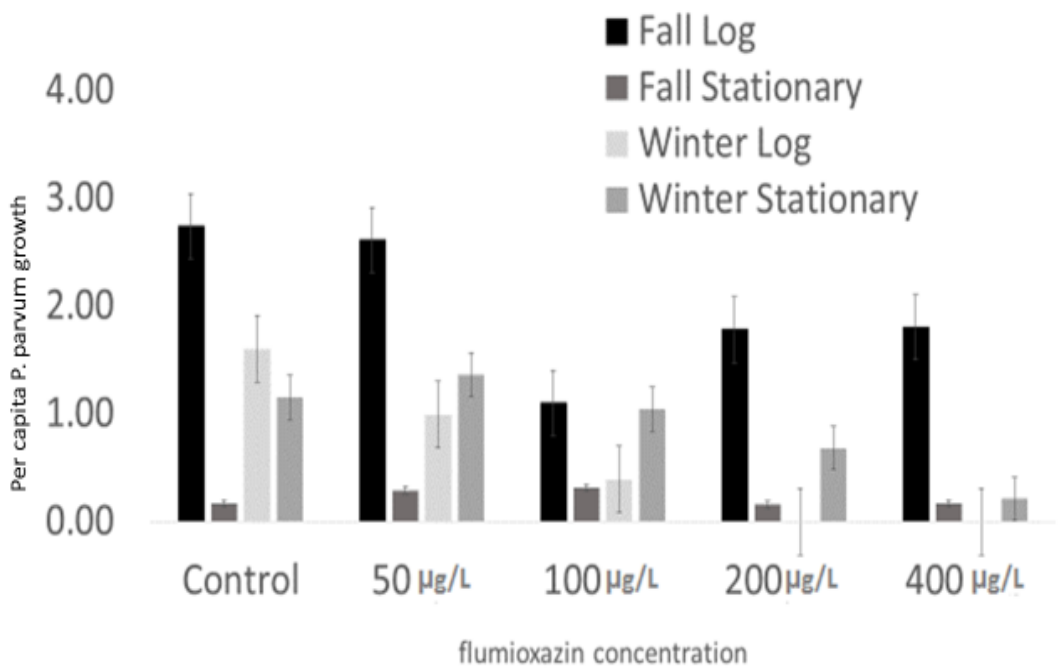

Fig. 3.

Per capita *P. parvum* growth after 12 days of exposure to various levels of Clipper. Clipper control of *P. parvum* is not significant in conditions of fall temperatures ($p>0.05$), but is significant in conditions of winter temperatures ($p<0.05$). Vertical bars depict standard deviation in data (n=3).

Fall- 16°C

Per capita cell reproduction rates had no significant (p interactions between growth stage and Clipper concentration. At every time step there is a significant ($p<0.05$) effect by the growth stage as a main effect.

Winter- 12°C

For the first 2 days of the screening, Clipper concentration was significant as a main effect on the per capita *P. parvum* growth. After the first two days, growth stage became a separate main effect

and then cell growth was affected by interactions between growth stage and Clipper concentration. (Tab. 4)

16°C	2	4	6	8	10	12
Interactions and Effects						
GS*Cconc	0.165	0.352	0.555	0.822	0.563	0.092
GS	0	0	0	0	0	0
Cconc	0.396	0.606	0.808	0.822	0.563	0.119
12°C						
Interactions and Effects						
GS*Cconc	0.487	0.764	0.041	0.045	0.04	0.02
GS	0.283	0	0.002	0	0	0.008
Cconc	0.002	0.001	0	0	0	0

Table 4. 2-way ANOVA *P. parvum* per capita daily growth. Data is divided along the lines of the effects of temperature. Values listed are the p values for per capita daily growth from the treatment with Clipper to the day listed at the top of the column (ti-t0). Highlighted squares are the interactions or effects considered significant (p value < 0.05 and prioritizing interactions before effects)

3.3 Effects on chlorophyll-*a*

Increasing Clipper concentrations was effective at achieving a decrease in chlorophyll-*a* fluorescence in winter temperatures regardless of growth phase. Concentrations higher than 200µg/L were successful at attaining negative change in chlorophyll-*a* fluorescence in winter conditions while *P. parvum* was in log growth phase. (Fig. 4)

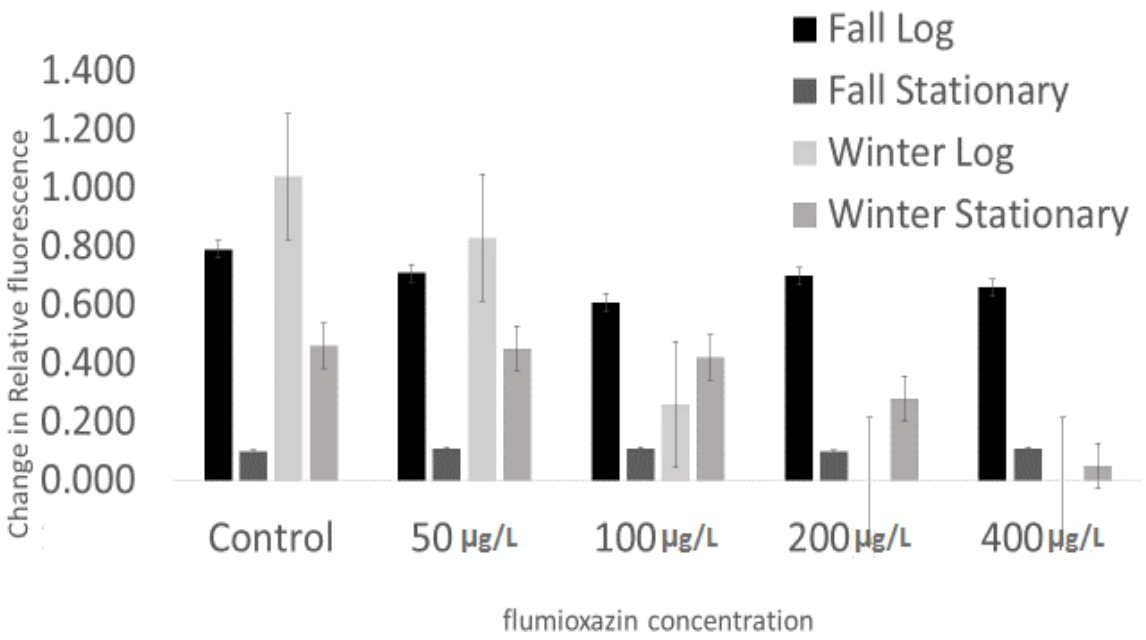

Fig. 4.

Change in relative fluorescence of *P. parvum* after 12 days of exposure to various levels of Clipper. Clipper control of *P. parvum* is not significant in conditions of fall temperatures ($p>0.05$), but is significant in conditions of winter temperatures ($p<0.05$). Vertical bars depict standard deviation in data (n=3).

The three-way interaction between the tested independent variables (Clipper concentration x season x growth stage) was significant in effect to the chlorophyll-*a*. This was true through the duration of the screening. (Tab. 5)

	Day					
	2	4	6	8	10	12
Interactions and Effects						
Temp*GS*Cconc	0.001	0	0	0	0	0
GS*Cconc	0	0	0	0	0	0
Temp*Cconc	0	0	0	0	0	0
Temp*GS	0	0	0	0	0	0
Temp	0	0	0	0	0.424	0.078
GS	0.003	0.011	0	0	0	0
Cconc	0	0	0	0	0	0

Table 5. 3-way ANOVA chlorophyll-*a* fluorescence. Values listed are the p values for chlorophyll-*a* relative fluorescence from the treatment with Clipper to the day listed at the top of the column (ti-t0). Highlighted squares are the interactions or effects considered significant (p value < 0.05 and prioritizing interactions before effects)

2-way- analysis of variance revealed that growth stage was the only significant effect in fall temperatures, but that in winter temperatures, growth stage began as the only significant variable, but the interaction between growth stage and Clipper concentration quickly became significant. By the close of the screening, growth stage became the sole significant variable.

Fall- 16°C

Changes in chlorophyll-*a* were not affected by interactions between growth stage and Clipper concentration. At every time step there is a significant effect by the growth stage as a main effect.

Winter- 12°C

For the first 2 days of the screening, growth stage affected changes in chlorophyll-*a* as a main effect. After the first two days, growth stage and Clipper concentrations interacted to impact chlorophyll-*a* until the close of the screening where growth stage, once again, as a main effect affected chlorophyll-*a*.(Tab. 6)

16°C	2	4	6	8	10	12
Interactions and Effects						
GS*Cconc	0.62	0.125	0.439	0.118	0.946	0.135
GS	0.001	0.018	0	0	0	0.002
Cconc	0.766	0.764	0.929	0.626	0.584	0.199
12°C						
Interactions and Effects						
GS*Cconc	0.439	0	0.009	0	0	0.051
GS	0	0	0	0	0	0
Cconc	0.512	0	0.086	0.001	0	0.053

Table 6. 2-way ANOVA Chlorophyll-*a* flourescence. Data is divided along the lines of the effects of temperature. Values listed are the p values for chlorophyll-*a* relative fluorescence from the treatment with Clipper to the day listed at the top of the column (ti-t0). Highlighted squares are the interactions or effects considered significant (p value < 0.05 and prioritizing interactions before effects)

4. DISCUSSION AND CONCLUSIONS

The effect of season by means of temperature differences on both the growth rate and the change of chlorophyll-a in *P. parvum* is consistently present for 12 days as observed in this study. However, other factors were involved as temperature was not a main effect in any case. Growth rates were affected by interactions between temperature and growth stage and between temperature and Clipper concentration. Change in chlorophyll-α was observed to be from an interaction between all three independent variables for all 12 days.

It was assumed that temperature would affect the efficacy of Clipper with the reasoning that higher temperatures produce faster chemical reactions (Matzenbacher et al. 2014). This led me to hypothesize that higher temperatures would allow Clipper to work more effectively at controlling *P. parvum.* However, the opposite of this was seen. At the fall temperature, Clipper concentration was not a significant factor at all in controlling reproductive rates of *P. parvum,* but at the winter temperature, the interaction of Clipper concentration and growth stage was a significant factor.

Hydrolysis of Clipper occurs at a more accelerated rate with increasing pH values. pH values of 5, 7 and 9 yielded half-lives of 16.4, 9.1, and 0.25 hours respectively in another study (Kwon et al. 2004). The range of pH values observed in the current study was 7.2 to 7.8. The lowest was at both growth stages in winter temperatures and the highest was at stationary growth in fall temperatures. While it follows that this study did show greater efficacy with lower pH, the effect was greater than should have been expected by the relatively narrow range of pH. It is unlikely that the differences in pH among temperatures could fully explain the observed effect of season on Clipper efficacy.

Differences in observed effects between seasons could be because of the differences in initial cell density in the study. (Franklin et al. 2002) showed how different initial cell densities affect the growth rates and final cell densities in bioassays conducted in laboratory settings. The increments between initial cell densities tested by Franklin et al. (2002) were greater than the margin of initial

cell densities in the current study. The cell densities tested varied within an order of magnitude, i.e., 8.8×10^3-4.5×10^4 cells/mL. Like the differences in pH, the difference in initial cell densities may play a part in explaining the observed differences in efficacy between seasons.

P. parvum growth rates can differ between temperatures. (Baker et al. 2007) cultured *P. parvum* at temperatures of 11°C and 15°C and found 15°C to be more optimal for growth. A growth rate high enough could have a greater effect on a population than the chemical acting to counter it. In log growth it would be expected that growth rates would be increasing through time while Clipper effects decreased allowing *P. parvum* to persist and grow in the presence of Clipper. Comparing growth rates from the control groups, the highest rates were seen at the fall temperature in log growth. This supports the notion that increased growth rates may overshadow the effects of Clipper on the population.

Clipper contains supposedly inert ingredients. One or more of these ingredients may not be so inert and may hinder the action of the active ingredient. Umphres et al. (2012) conducted a similar study using flumioxazin without added ingredients and found a significant effect on *P. parvum* fluorescence as did the current study, so it is not likely that an inert ingredient interfered with the activity of flumioxazin unless one or more of these ingredients become active under certain conditions that include a threshold temperature between 12°C and 16°C used in this study.

Umphres et al. (2012) suggested that chemical applications at earlier growth phases (log phase) could be effective at controlling *P. parvum* blooms long enough for bloom conditions to subside. This idea seems likely in light of the results of this study, but efficacy is also dependent on the season. If applications are made proactively, then there would be an advantage due to the log growth stage of *P. parvum*, but if temperatures are still too warm, then effective doses will increase.

Flumioxazin applications in the ready to use formula sold under the name of Clipper can be used to effectively control *P. parvum*, but considerations must be made to temperature. Consideration

of the growth stage of *P. parvum* would be of further benefit. This study indicates that the most effective application in the southeastern United States would be made in winter (or cooler temperatures around 12°C) while *P. parvum* is in log growth. Under these conditions, applications of 200 μg/L or greater could even create a negative growth rate in the population and this concentration would be necessary for an effect to be seen in stationary growth stages.

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